**16. Assessing mitochondrial bioenergetics in isolated mitochondria from various mouse tissues using Seahorse XF96 analyzer.**

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**Summary**

Working with isolated mitochondria is the gold standard approach to investigate the function of the electron transport chain in tissues, free from the influence of other cellular factors. In this chapter, we outline a detailed protocol to measure the rate of oxygen consumption (OCR) with the high-throughput analyzer Seahorse XF96. More importantly, this protocol wants to provide practical tips for handling many different samples at once, and take a real advantage of using a high-throughput system. As a proof of concept, we have isolated mitochondria from brain, heart, liver, muscle, kidney, and lung of a wild type mouse, and measured basal respiration (State II), ADP-stimulated respiration (State III), non-ADP-stimulated respiration (State IVo), and FCCP-stimulated respiration (State IIIu) using respiratory substrates specific to the respiratory chain complex I (RCCI) and complex II (RCCII). Mitochondrial purification and Seahorse runs were performed in less than 8 working hours.

**Key Words**: High-throughput, Seahorse XF96, mitochondrial coupling assay, respiratory control ratio (RCR), mouse tissues

1. **Introduction**

To investigate the function of the electron transport chain in animal tissues there are two approaches. One approach is to measure the rate of oxygen consumption (OCR) directly in small biopsies permeabilized with detergents; the other is to isolate mitochondria from the tissue of interest and measure respiration in an isotonic buffer supplemented with respiratory substrates and ADP. When working with permeabilized tissues there are many variables influencing the measurement, such as the accessibility of substrates and inhibitors throughout the tissue, the limited diffusion of oxygen (O2), the coexistence of various types of cells, the variable number of mitochondria per cell, the availability of endogenous substrates, and the rate of ATP utilization. Moreover, effective protocols to measure O2 consumption directly in permeabilized tissues are available only for a few tissues [[1-3](#_ENREF_1)]. Instead, working with isolated mitochondria has the advantage of disconnecting the mitochondrial respiration from cellular factors. In isolated mitochondria the OCR relies only on the activity of the RCCs and on the substrates and ADP exogenously added to the system.

Measuring oxygen consumption in isolated mitochondria is not a new concept. This is carried out routinely by traditional O2 electrode-based methods [[4](#_ENREF_4),[5](#_ENREF_5)]. However, those systems require a large amount of mitochondria for each measurement, between 0.01 and 0.5 mg, depending on the tissue, and long running times, between 1-2 hours, per replicate. Large amount of mitochondria and the challenge to perform a significant number of replicates in a timely manner to preserve mitochondrial coupling represent a major limitation when working with precious biological materials. The advance of high- throughput microplate respiratory measurements has overcome this limitation [[6-8](#_ENREF_6)]. The protocol described in this chapter uses only 0.3-4 μg of mitochondria per measurement (i.e. per well) and it allows to perform at least 4 to 6 replicates for each sample in only 70 minutes. We will indicate how to quickly isolate mitochondria from brain, heart, liver, muscle, kidney and lung of a wild type mouse and how to measure OCRs for State II, State III, State IVo and Sate IIIu. Furthermore, we suggest how to analyze the Seahorse data to get values of respiratory control ratios (RCRs), as an indicator of mitochondrial coupling.

1. **Materials**
   1. **Equipment**

Homogenizer EUROSTAR 20 digital IKA 0004442000

Potter-Elvehjem PTFE pestle and glass tube Sigma P7859-1EA

Centrifuge Sigma 4K15

Plate rotor QIAGEN 09100

Centrifuge Sigma 6K15

Swing-out rotor Sigma 11150

Seahorse XF96 analyzer Seahorse Biosciences, Inc.

Inverted Fluorescent Microscope Leica DM IRE2

* 1. **Consumables**

|  |  |  |  |
| --- | --- | --- | --- |
| 15ml Polypropylene Tubes | | Falcon | 352059 |
| ADP | | Sigma-Aldrich | A2754 |
| Antimycin A | | Sigma | A8674 |
| D-Mannitol | | Sigma-Aldrich | M9546 |
| Dulbecco's Phosphate buffered saline | | Gibco | 14190-094 |
| EGTA | | Sigma-Aldrich | E3889 |
| Fatty acid-free BSA | | Sigma | A3803 |
| FCCP | | Sigma | C2920 |
| Glutamate | | Sigma-Aldrich | G8415 |
| HEPES | | Sigma-Aldrich | H3784 |
| Hydrochloric acid 37% (HCl) | | Merck | 1E+09 |
| KH2PO4 | | Sigma | P9791 |
| Magnesium chloride (MgCl2) | | Sigma-Aldrich | M9272 |
| Malate | | Sigma-Aldrich | M6413 |
| Mito Tracker® Green FM | | Molecular Probes | M-7514 |
| Oligomycin | | Sigma | O4876 |
| Pasteur Pipettes | | BRAND | 7477-55 |
| Petri dish 60x15 | | Nuc | 150288 |
| Potassium chloride (KCl) | | Sigma-Aldrich | P9541 |
| Potassium hydroxide (KOH) | | Sigma-Aldrich | 221473 |
| Protein Assay Dye Reagent Concentrate | | Bio-Rad | 500-0006 |
| Rotenone | | Sigma | R8875 |
| Succinate | | Sigma-Aldrich | S2378 |
| Sucrose | | Sigma | S0389 |
| Surgical Disposable Scalpels | | BRAUN | BBA215 |
| Tween-20 | | Sigma | P9416 |
| XF96 Cell Culture Microplate | | Seahorse Bioscience | 100850-004 |
| XF96 Sensor Cartridge | | Seahorse Bioscience | 102416-100 |
|  |  | |  |

* 1. **Buffers**

1. Prepare the mitochondrial isolation buffer (MIB1), by dissolving 210 mM of D-Mannitol, 70 mM of sucrose, 5 mM of HEPES, 1 mM of EGTA and 0.5% (w/v) of fatty acid-free BSA in ultrapure H2O and adjust the pH to 7.2 with KOH. Prepare aliquots and store them at -20°C (*see* **Note 1**).

2. Prepare the mitochondrial assay solution (MAS1) by dissolving 220 mM of D-Mannitol, 70 mM of sucrose, 10 mM of KH2PO4, 5 mM of MgCl2, 2 mM of HEPES, 1 mM of EGTA and 0.2 % (w/v) of fatty acid-free BSA in ultrapure H2O and adjust the pH to 7.2 with KOH at 37°C. Prepare aliquots and store them at -20°C (*see* **Note 1**).

* 1. **ADP and respiration reagents**
     1. Prepare mitochondrial substrates by dissolving 0.5 M of succinate, 0.5 M of malate, 0.5 M of glutamate in ultrapure H2O and adjust the pH to 7.2 at 37°C with KOH. Prepare aliquots and store at -20°C (*see* **Note 2**).
     2. Prepare ADP by dissolving 40 mM of ADP in MAS1 and adjust the pH to 7.2 with KOH. Perform serial dilutions to obtain stocks of 20 mM, 10 mM, 5 mM, 2.5 mM, and 1.25 mM of ADP in MAS1 (*see* **Note 3**).
     3. Prepare stocks of inhibitors and uncouplers by dissolving 10 mM of FCCP, 2 mM of rotenone, 5 mg/ml of oligomycin and 40 mM of antimycin A in DMSO. Prepare aliquots and store at -20°C. Prevent freezing and thawing of Rotenone and FCCP stocks. On the day of the experiment, dilute them with MAS1 to working concentrations of 40 µM (*see* **Note 4**), 20 mM, 25 µg/ml, and 40 mM for FCCP, rotenone, oligomycin, and antimycin A, respectively.

It is recommended to thaw all reagents on ice and keep them on ice during the whole experiment.

1. **Methods**
   1. **Rehydrating the cartridge (Day before the experiment)**
      1. Hydrate the XF96 Sensor Cartridge by adding 0.2 ml of XF calibrant in each well of the utility plate (lower transparent plastic plate).
      2. Carefully place the cartridge (upper green plate containing ports) back onto the utility plate (fluorophores at the bottom of the cartridge can be easily damaged).
      3. Incubate the plate in a non CO2 incubator at 37°C over-night (*see* **Note 5**).
   2. **Set up the experimental protocol in the Seahorse XF96 analyzer** **(Day before the experiment)**
      1. Open the Seahorse XF96 software (*see* **Note 6**).
      2. Select “Standard” assay.
      3. Wait for the initialization of the system.
      4. Go to “Assay Wizard” to fill in the project information under “General”.
      5. Fill in the information concerning each mitochondrial sample under “Cell information”. Select “Cell Layout” submenu to define how mitochondrial samples will be seeded in the 96-well plate (*see* **Fig. 1**). Assign a different color to each group in the “Groups & Labels” submenu.



Fig. 1. Layout of the 96-well Seahorse plate for the optimization of the mitochondrial amount. Up to three different amounts of mitochondria for a specific substrate and tissue can be tested by using this layout. Each measurement is carried out in quadruplicate. The last two rows are filled in with MAS1 and represent the background (BK).

* + 1. Fill in the information concerning the run under “Protocol”. You can adjust the parameters by selecting the commands on the display. The protocol used to test mitochondrial coupling is summarized in **Table 1**
    2. Save the file as template (.xls). Unless specified, the file will be automatically saved in the folder XFReader96.
    3. End wizard.

Table 1. Experimental outline to test mitochondrial coupling (*see* **Note 6**).

**3.3 Preparation of mouse organs (Day of the experiment)**

1. Mice are killed via instant cervical dislocation (see **Note 7**) without prior anesthesia such as CO2, since this influences mitochondrial respiration.
2. Subsequent organ withdrawal should be completed within max. 30 minutes per mouse and each organ is kept in a different Petri dish on ice upon withdrawal.
3. If necessary, carefully remove hair and fat residues from organs, as these interfere with the subsequent isolation of mitochondria.
   1. **Isolation of mouse mitochondria (Day of the experiment)**

This protocol can be applied to isolate mouse mitochondria from liver, heart, lung, brain, kidney and hindlimb skeletal muscle tissues. Start with 30-50 mg wet-weight tissue**.**

1. Prepare on ice six Petri dishes with 1ml of MIB1.
2. Place each tissue in a different Petri dish.
3. Cut tissues into very small pieces with disposable scalpels (*see* **Note 8**).
4. Collect pieces with a disposable 1ml plastic Pasteur pipette and place them in 15ml polypropylene tubes containing 5 ml of MIB1.
5. Transfer the suspension into the glass tube of the Potter-Elvehjem PTFE for homogenization.
6. Disrupt tissue pieces by 30 strokes at 500 rpm in the Homogenizer EUROSTAR 20 digital, except the muscle which is homogenized at 600 rpm.
7. Centrifuge the homogenate at 800 g for 10 min at 4°C in a swing-out rotor.
8. Collect the supernatant.
9. Centrifuge the supernatant at 8000 g for 10 min at 4°C in a swing-out rotor.
10. Discard the supernatant.
11. Carefully remove fat and lipids from the pellet, especially in the liver sample (*see* **Note 9**)
12. Wash the pellet twice with MIB1.
13. Resuspend the final pellet, which contains mitochondria, in a small volume of MAS1 containing the appropriate respiratory substrate. Use 10mM of Glutamate and 5mM Malate for complex I-driven respiration, and 10 mM Succinate for Complex II (*see* **Note 10**).
14. Determine the total protein amount by Bradford assay (*see* **Note 11**).
    1. **Loading the cartridge with compounds and run the calibration (Day of the experiment)**
15. Load 10-fold concentrated compounds in the ports of the cartridge, with the help of the “loading helper” plate. Inject 20 μl of ADP (40 mM) (*see* **Note 12**) into port A, 22 μl of oligomycin (25 µg/ml) into port B, 24 µl of FCCP (40 µM) into port C and 26 μl of antimycin A (40 µM) or rotenone (20 µM) into port D.
16. Open the folder XFReader96 in the Seahorse machine.
17. Select the files “XF Data & Template.xls”.
18. Select the appropriate template file and set it as “default assay” for the subsequent run.
19. Click “Run”.
20. Insert the XF96 Sensor Cartridge into the Seahorse XF96 analyzer and hit ‘start’ to run the calibration. Calibration run takes around 30 min.
    1. **Testing of optimal mitochondrial amounts (Day of the experiment)**

The amount of mitochondria to use in the assay varies according to the tissue of origin, the respiratory substrate (*see* **Note 13**), and the purity and intactness after the isolation. Therefore, it is recommended to run an initial plate on the day of experiment to test the optimal mitochondria amount to use in the subsequent experiments. To this goal, run a mitochondrial coupling test experiment using glutamate/malate or succinate/rotenone as respiratory substrates.

* + 1. Dilute all mitochondria to 1 µg/µl with MAS1 containing the appropriate respiratory substrate.
    2. Prepare three different master mixes for each tissue, containing various amounts of mitochondria/well. Consider to use larger volumes for the master mixes, to minimize pipetting errors. For example:

*Mix 0.5µg*🡺 in 250µl of mixture, add 2.5 µl of mitochondria (1µg/µl) and 247.5 µl of MAS1 with substrates

*Mix 1 µg* 🡺 in 250µl of mixture, add 5 µl of mitochondria (1µg/µl) and 245 µl of MAS1 with substrates

*Mix 2µg* 🡺 in 250µl of mixture, add 7.5 µl of mitochondria (1µg/µl) and 242.5 µl of MAS1 with substrates

Keep the mitochondrial samples and solutions on ice.

Clean the outer surface of the pipette tip after aspiring mitochondria and before adding it to the MAS1 buffer. Ensure that mitochondrial samples are well resuspend to minimize variations in the measurements.

* + 1. Deliver mitochondria as a 50 µl suspension to each well of the plate (*see* **Figure 1**; *see* **Note 14**), except the wells intended for the background correction. In these wells add only MAS1 with substrates. Keep the plate on ice while pipetting. The background correction is monitoring changes in O2, pH and temperature due for example to the injection step and it is used to correct the measurement values from this noise. Therefore, different injection strategy need an independent background correction. Unfortunately, the wave program still misses the possibility to sort the background wells to specific wells in case you measure several conditions within the same plate. This should be carried out by the user during data analysis.
    2. Transfer the plate to a centrifuge with a swinging bucket microplate adaptor. Spin down the mitochondria to the bottom of the plate by centrifuging the plate at 2000 g for 20 min at 4°C.
    3. During the 20 minutes of centrifugation, start the calibration plate in the Seahorse machine (*see* **Paragraph 3.3.4**).
    4. After the centrifugation, inspect mitochondria briefly under the microscope to ensure consistent adherence to the bottom of the wells (*see* **Note 15**).
    5. Add 130 µl of pre-warmed (37°C) MAS1 with substrates (glutamate/malate or succinate/rotenone) to each well.
    6. Warm up the plate at 37°C in a non-CO2 incubator for 5-10 minutes.
    7. At this stage the calibration step should be ready. Remove the utility plate from the machine. The cartridge is automatically retained in the instrument.
    8. Put the microplate in the analyzer and start the run at the end of the calibration (*see* **Note 16**).
    9. At the end of the run, open the .xfd file, select Display>Group, Y1>Level, Y2>O2. The optimal mitochondrial amount is the amount that never makes the absolute O2 tension decline to zero, especially in State III or State IIIU (*see* **Note 17**).
  1. **Run of the experiment using the optimal mitochondria amount (Day of the experiment)**

Test mitochondrial coupling as described in Paragraph 3.4, using the proper mitochondria amount for each tissue.

* 1. **Verification of mitochondrial integrity after the run (Day of the experiment)**
     1. After the run, recover the plate from the machine.
     2. Dispense ~180 µl of MAS + BSA, 1X buffer + 40 nM MitoTracker Green FM.
     3. Incubate 15 minutes at 37°C
     4. Acquire pictures with an inverted fluorescent microscope (*see* **Figure 2**). Intact mitochondria are able to uptake the probe, therefore fluoresce.



Fig. 2. Isolated mitochondria remain attached to the plate and coupled throughout the assay.The figure shows a representative picture of mitochondria isolated from heart, plated at 1 µg/well on the XF96 cell culture microplate and stained with 20 nM of MitoTracker Green (+MTG). The fluorescent signal in the pictures +MTG indicates that mitochondria are still able to uptake the probe, suggesting that the mitochondrial membrane potential is still intact. Pictures were acquired using an inverted microscope with 20X magnification.

**3.9 Data analysis (Any day after the experiment)**

1. Open the .xfd files with the XFeWave software.
2. Select Modify>Normalization>Apply to normalize the OCR values of each well to ug of mitochondria.
3. Select Display>Well, Y1>Rate, Y2>None to visualize outliers due to incorrect injection of reagents or uneven distribution of mitochondria and exclude them from the analyses by clicking on the trace.
4. Select Display>Group, Y1>Rate, Y2>None.
5. Right click on the line chart area and export graph data in an Excel sheet. You will get a list of OCR values, expressed as pmol 02/min/µg mitochondrial proteins, at different time points (1, 6, 13, 20, 25, 31, 35 minutes) and the S.E.M. values.
6. Calculate the basal respiration, which represents the respiration of mitochondria in presence of substrates but without ADP, by subtracting the OCR values at minute 35 (non-mitochondrial O2 consumption) from the values at minute 1 (see **Figure 3**).
7. Calculate State III, which represent the formation ATP from ADP and inorganic phosphate, by subtracting the OCR values at minute 35 from the values at minute 13 (*see* **Figure 5** for calculation**;** *see* **Figures 4** and **5** forreference values).
8. Calculate State IVo, which represents the proton leak due to the inhibition of the ATP synthase by oligomycin, by subtracting the OCR values at minute 35 from the values at minute 20 (*see* **Figure 3** for calculation**;** *see* **Figures 4** and **5** forreference values).
9. Calculate the state IIIu, which represents the status of maximal respiratory capacity, by subtracting the OCR values at minute 35 from the values at minute 25(*see* **Figure 3** for calculation**;** *see* **Figures 4** and **5** forreference values).
10. Calculate the respiratory control ratio, RCR, which is the index of mitochondrial coupling, by dividing the corrected values of State IIIu/State IVo (*see* **Figures 4** and **5** for reference values).



Fig. 3. Representative trace of a coupling experiment using isolated mitochondria.



RCRADP 5,0 4,0 20,0 3,4 6,1 3,8

Fig. 4. OCR and RCR for RCCI-driven respiration. Data refer to mitochondria isolated from wild-type C57Bl/6N mice, 14-19 weeks-old. Data represent the average of three independent experiments, each experiment having 4-6 replicate wells +SEM (9).



RCRADP 6,4 6,4 6,7 3,7 3,5 3,0

Fig. 5. OCR and RCR for RCCII-driven respiration. Data refer to mitochondria isolated from wild-type C57Bl/6N mice, 14-19 weeks-old. Data represent the average of three independent experiments, each experiment having 4-6 replicate wells +SEM (9).

**5. Notes**

1. Buffers can be prepared up to 2-3 weeks in advance and aliquots stored at -20°C. However, on the day of the assay, the pH has to be remeasured and eventually readjusted to 7.2 with KOH.
2. Substrates are stable for long periods (> 1 year) at -20°C.
3. 40mM of ADP and serial dilutions should be prepared on the day of the assay or at the earliest, one the day before.
4. The concentration of FCCP depends on the BSA content in the MAS buffer, the amount of mitochondria, and the type of mitochondria. If using a different concentration of BSA in the MAS buffer, or using mitochondria isolated from tissues not described in this protocol, a preliminary FCCP titration is mandatory.
5. The hydration step is required for proper functioning of the sensors when measuring OCR. Cartridges can be used within 3–4 hours of hydration, as well as up to 72 hours after the addition of the calibrant solution. The incubator must be humidified if you are hydrating for longer periods of time to prevent the evaporation of the calibrant solution. Remember to hydrate as many cartridges as required for the whole experiment.
6. The protocols described here is adapted for the XF Reader version 1.4.2.3. Be aware that latest versions might have different user interfaces.
7. Extend measurement times for rotenone and antimycin A to 6 minutes because those measurements are going to be used as “not mitochondrial respiration”, hence background, to be subtracted from all respiratory states.
8. Attain to your local laws for animal handling. In our experiments mice are sacrificed by cervical dislocation in accordance with the German Animal Welfare Act (§7, §8 and §8a).
9. Rinse liver, heart and kidney several times with MIB1 to remove blood.
10. Use a cotton swab to remove fat and lipids from the outer part of the mitochondrial pellet. You may recognize this fraction from the typical whitish/pale-yellow color.
11. Choose the volume according to the size of the pellet. Usually, 100 µl for muscle and lung, 200 µl for brain, 500 µl for liver and kidney, if starting from 30-50 mg of tissue.
12. Prepare the reagent “blank” with MAS1 since BSA in the buffer will interfere with the Bradford measure of your samples.
13. Using an appropriate concentration of ADP is essential for determining OCR during State III. The optimal ADP concentration should not be necessarily determined on the day of the assay. It can be measured at any time before the assay, by using isolated mitochondria from a wild type mouse. We tested ADP concentrations in the range of 0.125 mM to 4 mM, as described in (6) and we found that most of the tissues included in this chapter were stimulated by 2-4 mM of ADP. If you need to test the correct ADP amount in a different tissue, we recommend to test mitochondrial coupling using glutamate/malate or succinate/rotenone as respiratory substrates at increasing concentrations of ADP, as described here:

* Distribute the optimal amount of mitochondria for two different tissues on the same plate.
* Inject different amounts of ADP through port A: 0 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM, 4 mM (*see* **Figure 6**). Proceed by loading ports B-D as described in **Paragraph 3.3**.



Fig. 6 Layout of the 96-well Seahorse plate for the optimization of the ADP concentration. Using the present layout you can test up to seven different concentrations of ADP for a specific substrate and tissue. Every measure is carried out in six replicates. The last row is filled with MAS1 and represents the background (BK).

* At the end of the run analyze the .xfd file and select the optimal ADP concentration as the concentration giving a clear increase in respiration, still responding to the oligomycin and the FCCP.
* The molarity of ADP to be injected is 10 times higher (10X) than the final molarity in the well. In our hands, a final concentration between 2 to 4 mM of ADP is sufficient to induce a stable State III. Insufficient amounts of ADP will result in the exhaustion of ADP during one measurement cycle.

1. As a rule of thumb, the amount of mitochondria to use for RCCI-dependent respiration is 3-4 times higher than the amount of mitochondria to use for RCCII-stimulated respiration. Perform pilot experiments for testing different concentrations of mitochondria for each tissue. Use a range of 0.5 to 2 µg mitochondria/well for succinate-dependent respiration and of 1 to 5 µg mitochondria/well for glutamate/malate-dependent respiration.
2. It is not recommended to use large suspension volumes as it will result in lower OCRs probably due to attached mitochondria at the sides of the wells.
3. Mitochondria should appear evenly distributed in each well.
4. Start the calibration run as soon as you start centrifuging the microplate with mitochondria.
5. A good indication is to keep the basal respiration between 40-80 pmol O2/min/well

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